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(54) Title: METHOD FOR GENERATING REPLACEMENT CELLS AND/OR TISSUES

(57) Abstract: Disclosed are methods of isolating differentiated cells or adult stem cells from pluripotent cells and particularly ICM cells, by exposing such pluripotent cells to environmental cues in order to encourage development along a certain path.

Method for Generating Replacement Cells and/or Tissues

Related Applications

[0001] This application claims benefit of priority to U.S. Serial No. 60/274,216 filed on March 9, 2001 and of which are incorporated by reference in their entirety herein.

Field of Invention

[0002] The present invention is concerned with engineering replacement cells and tissues that may be used for transplantation into patients in need of such tissues. In particular, the present invention provides a means for producing isogenic replacement tissues using nuclear transfer, without requiring *in vitro* isolation and differentiation of embryonic stem cells.

Background of the Invention

[0003] Embryonic stem (ES) cell technology is one of the few known means for generating large numbers of replacement cell types from pre-implantation stage embryos. Nuclear transfer technology in particular enables the isolation of ES and inner cell mass (ICM) cells from a differentiated somatic cell nuclear donor. This technology provides great promise for engineering cells and tissues for human transplantation, because such tissues can be designed using the patient's own cells as the source of nuclear DNA, thereby avoiding the problems of transplant rejection and graft versus host reactions associated with transplanting allogeneic and xenogeneic tissues.

[0004] Despite the promise of this technology, many years of research may still be required before biologists understand how to direct ES cells *in vitro* along a desired line of development in order to generate replacement cells and tissues for human patients. Furthermore, it may be ethically and legally problematic to implant human and/or chimeric human-animal embryos into animals, or to implant human and/or chimeric human-animal ICMs/ES cells into animals that may have the capacity to generate embryos and/or teratomas. Thus, there is an immediate need for methods that enable isolation of

differentiated cells and tissues from ES cells or other pluripotent stem cells that do not require a knowledge of the developmental signals that guide embryonic development, and that do not raise ethical and legal dilemmas with regard to human patients.

[0005] In this regard, researchers have shown using an *in utero* xenotransplantation approach that neural progenitor cells from mice differentiate into cells having glial-like features after injection into the rat forebrain ventricle. See Winkler et al. (June 1998) Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain, Mol. Cell. Neurosci. 11(3): 99-116. Similarly, human neural precursor cells that had been expanded *in vitro* were shown to develop into neurons in a site-specific manner after being transplanted into either an adult or neonatal rat brain. See Fricker et al. (July 1999) Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain, J. Neurosci. 19(14): 5990-6005; see also Rosser et al. (July 2000) The morphological development of neurons derived from EGF- and FGF-2-driven human CNS precursors depends on their site of integration in the neonatal rat brain, Eur. J. Neurosci. 12(7): 2405-13. In these studies, the resulting neuron cells were not purified but rather were traced by mouse-specific and human-specific markers. Nevertheless, these results suggest that neural progenitor cells respond to host-derived environmental signals that direct their differentiation along multiple phenotypic pathways.

[0006] Researchers from the University of South Florida have recently extended this premise to an *in vitro* setting by showing that it is possible to induce human or mouse bone marrow stromal cells (BMSC), which normally give rise to bone, cartilage, and mesenchymal cells, to differentiate into neuron-like cells by culturing them in the presence of rat fetal mesencephalic or striatal cells. See Sanches-Ramos et al (August 2000) Adult bone marrow stromal cells differentiate into neural cells *in vitro*, Exp. Neurol. 164(2): 247-56. Thus, it may be possible to mimic the environmental signals that induce pluripotent cells to differentiate along a given pathway *in vitro* merely by exposing

pluripotent cells to differentiated cells. However, it is not clear how useful cellular engraftment will be in repairing damaged tissues absent tissue engineering, therefore, differentiated cells derived *in vitro* still have limited utility.

[0007] Furthermore, in order to employ cells that have been environmentally induced to differentiate along a certain developmental path, one must have a means of isolating or purifying such cells away from the inducing cells. This is particularly true where human cells are induced to differentiate in the presence of rat cells or other animal cells that might harbor viruses or other infectious agents that could potentially present a danger to human patients receiving such replacement cells. In this regard, Klug et al have described an approach for isolating relatively pure cardiomyocytes from differentiating murine embryonic stem (ES) cells whereby ES cells are stably transfected with a fusion gene consisting of a selectable marker, aminoglycoside phosphotransferase, linked to the alpha-cardiac myosin heavy chain promoter. See Klug et al (July 1996) Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intercardiac grafts, J. Clin. Invest. 98(1): 216-24; see also McWhir et al (Oct 1996) Selective ablation of differentiated cells permits isolation of embryonic stem cell lines from murine embryos with a nonpermissive genetic background, Nat. Genet. 14(2): 223-6 (using a similar technique to select against differentiated cells). The aminoglycoside phosphotransferase-transfected cells in Klug were differentiated *in vitro* and subjected to G418 selection, and the resulting cardiomyocyte cultures were shown to be highly pure (>99%). Further, stable ES-cell derived cardiomyocyte grafts were observed in the hearts of adult mice seven weeks after implantation. However, as noted by Klug in this report, cellular engraftment has questionable utility in effecting myocardial repair.

[0008] Thus, there is still a need for methods which allow the production and isolation of differentiated cells, adult stem cells and tissues for replacement therapy. Moreover, it would be advantageous to design such replacement cells to genetically match the patient to avoid rejection of the cells and tissues and other harmful immune responses following transplantation into the patient.

Brief Description of the Figures

[0009] Figure 1. Illustration of renal unit. Renal cells were expanded in vitro, and then seeded onto collagen-coated cylindrical polycarbonate membranes. Devices with collecting systems were constructed by connecting the ends of three membranes with Silastic catheters that terminated in a reservoir made from polyethylene.

[0010] Figure 2. Tissue-engineered renal units retrieved 3 months after implantation. A. Unseeded control. B. Seeded with allogeneic control cells. C & D. Seeded with cloned cells, showing the accumulation of urinelike fluid.

[0011] Figure 3. Characterization of tissue retrieved 12 weeks after implantation. A. Cloned cells stained positively with synaptopodin antibody (A) and AQP1 antibody (B). The allogeneic controls displayed a foreign body reaction with necrosis (C). Cloned explant shows organized glomeruli (D) and tubule (E)-like structures. H&E, reduced from 400x. Immunohistochemical analysis using factor VIII antibodies identifies vascular structure within D (F). Reduced from x400.

[0012] Figure 4. RT-PCR analyses (upper) confirming the transcription of AQP1, AQP2, Tamm-Horsfall protein and synaptopodin genes exclusively in the cloned group (Cls). Western blot analysis (lower) confirms high protein levels of AQP1 and AQP2 in the cloned group, whereas expression intensities of CD4 and CD8 were significantly higher in the control allogeneic group (Co1&2).

[0013] Figure 5. Retrieved muscle tissues: A. Cloned cardiac muscle tissue retrieved shows a well-organized cellular orientation 6 weeks after implantation. H & E, reduced from 200x. B. Immunocytochemical analysis using troponin I antibodies identifies cardiac muscle fibers within the implanted constructs 6 weeks after implantation. Reduced from 200x. C. Cardiac muscle cell implant in control group shows fibrosis and necrotic debris in 6 weeks. H & E, reduced from 100x. D. Cloned skeletal muscle cell implants shows well-organized bundle formation. H & E, reduced from 40x. E. Retrieved skeletal muscle cell implant with polymer fibers. H & E, reduced from 200x. F.

Immunohistochemical analysis using sarcomeric tropomyosin antibodies identifies skeletal muscle fibers within the implanted second-set constructs 12 weeks after implantation. Reduced from 40x. G. Retrieved cloned skeletal muscle cell implants show spatially oriented muscle fiber 12 weeks after implantation. H & E, reduced from 100x. H. Retrieved control skeletal muscle cell implant shows fibrosis with increased inflammatory reaction in 12 weeks. H & E, reduced from 40x. I. Skeletal muscle cell implant in control group shows an increased number of inflammatory cells, fibrosis, and necrotic debris in 12 weeks. H & E, reduced from 100x. J. Immunocytochemical analysis using CD4 antibodies identifies CD4+ T cells within the implanted control cardiac muscle construct 6 weeks after implantation. Reduced from 100x.

[0014] Figure 6. Semi-quantitative RT-PCR products indicate specific mRNA in the retrieved tissues. A. Skeletal muscle tissue. Mean expression intensities of myosin and GAPDH (myosin/GAPDH) were calculated with NIH image software (CO 6; 0.02, CL 6; 0.22, CO 12; 0.00, CL 12; 0.09). B. Cardiac muscle tissue. Mean intensities of troponin I and GAPDH (troponin T/GAPDH) were calculated (CO 6; 0.00, CL 6; 0.15, CO 12; 0.03, CL 12; 0.29). CO 6; the control group at 6 weeks, CL 6; the cloned group at 6 weeks, CO 12; the control group at 12 weeks, CL 12; the cloned group at 12 weeks.

[0015] Figure 7. Western blot analysis of the implants confirmed the expression of specific proteins in the skeletal and cardiac muscle tissues. A. Skeletal muscle tissues. Mean expression intensities of desmin were calculated with NIH image software (CO 6; 30.58, CL 6; 85.14, CO 12; 52.61, CL 12; 79.48). B. Cardiac muscle tissues. Mean expression intensities of troponin I were calculated (CO 6; 23.17, CL 74.62, CO 12; 54.23, CL 12; 93.75). Mean expression intensities of desmin (CO 6; 16.08, CL 6; 67.81, CO 12; 52.41, CL 12; 121.39). CO 6; the control group in 6 weeks, CL 6; the cloned group at 6 weeks, CO 12; the control group at 12 weeks, CL 12; the cloned group at 12 weeks.

Summary of the Invention

[0016] The present invention solves the problems of the prior art by providing a method whereby differentiated cells and adult stem cells may be generated *in vivo*, by exposing nuclear transfer-generated ICM cells and other pluripotent embryonic cells to the appropriate cellular or tissue-type environment to encourage development of said pluripotent cells along a desired path. Chemical selection can be accomplished *in vivo* to encourage the formation of cohesive tissues, and may also be used at the isolation and purification stage to separate the cloned cells away from the cells of the host.

[0017] For instance, ICM cells can be injected into adult animals (i.e., SCID or nude mice), or more preferably into animal-embryos or fetuses at various stages of development. These cells could also be implanted into different sites to encourage differentiation into certain cell lineages. Injecting/implanting pluripotent stem cells into fetal environments may foster and encourage the cells to differentiate into cell types, such as pancreatic beta cells, that may not occur efficiently or completely in adult animals or in embryoid bodies *in vitro*. Starting chemical selection hours or days after injection or implantation of the cells is particularly important for generating human replacement cells and tissues, because this will eliminate the ethical/legal fear that a human baby or other human-animal entity will develop in the host animal. Furthermore, the sooner the growth and differentiation of the human pluripotent cells in a host mammalian embryo or fetus is restricted, the further replacement cell derivation can proceed without disrupting fetal development and growth.

[0018] The methods of the invention also encompass a multi-level targeted differentiation approach, whereby cells may first be encouraged to develop along a particular cell lineage path, such as an endodermal, mesodermal or ectodermal cell lineage, by controlled expression of markers specific for a particular lineage path. At another level, specific cell types and tissues may be isolated from lineage specific partially differentiated cells. For instance, cells can be directed down an endodermal path, from which islets may be then be isolated. Such a multi-level approach has the advantage of focusing a majority

of the implanted or injected cells in the desired direction, and facilitates purification of cells following differentiation into the desired cell type.

Detailed Description of the Invention

[0019] The present invention provides methods for encouraging the development of pluripotent cells along a particular path of differentiation and development by exposing such cells to environmental cues. Preferred pluripotent cells of the present invention are inner cell mass (ICM) cells, wherein such cells include cells derived or isolated from an ICM that have partially differentiated although they are still pluripotent. ES cells and other pluripotent cells may also be used. For instance, the invention includes a method of producing replacement cells and/or tissues for a mammal in need of such replacement cells and/or tissues, comprising:

(a) isolating an embryonic pluripotent cell or cells;

(b) introducing into said embryonic pluripotent cell(s) a selectable marker operatively linked to a cell or tissue specific promoter, enhancer or other regulatory genetic element such that said selectable marker is expressed only in the cell or tissue type of interest;

(c) permitting said embryonic cell(s) to differentiate into differentiated cells and tissues; and

(d) selecting for cells and tissues that express said selectable marker in order to produce replacement cells and/or tissues.

[0020] Preferably, the pluripotent cells employed in the present invention are human pluripotent cells. Such cells may be isolated using nuclear transfer of a human somatic cell nucleus into a mammalian enucleated oocyte or other suitable recipient cell using methods known in the art. In this regard, cross-species nuclear transfer is disclosed in PCT/US99/04608 and PCT/US00/05434 both of which are herein incorporated by reference in its entirety. Such methods as applied to human embryonic pluripotent cells are useful for generating replacement cells and tissues to be used for transplantation and to treat various diseases, i.e., heart disease, cancer and diabetes to name a few.

[0021] Replacement cells and/or tissues are any desired cell type, but are preferably selected from the group consisting of pancreatic beta cells, brain cells, neurons, cardiomyocytes, fibroblasts, skin cells, liver cells, kidney cells and islets. Alternatively, pluripotent cells can be induced to differentiate along certain development pathways, i.e., endodermal lines, mesodermal lines and ectodermal lines. Specific cell types and adult stem cells could then be isolated from a particular line of partially differentiated cells. For instance, islets could be isolated by encouraging further differentiation of an endodermal line of partially differentiated cells. Alternatively, nerve stem cells or hematopoietic stem cells or other adult stem cells could be obtained which have the potential to differentiate into a variety of cell types.

[0022] The pluripotent cells of the invention may be placed into a developing mammal at any appropriate age to facilitate directed differentiation and development. The cells are generally placed in the vicinity of the cell or tissue type desired. For instance, to encourage cardiomyocyte development, the cells may be placed into the heart muscle wall of the developing fetus. Cells may be implanted or injected as a mixture of individual cells, or could be arranged onto a synthetic scaffold or other extracellular matrix material using tissue engineering techniques. For instance, cells to be induced to develop into cardiomyocytes can be arranged on a scaffold and patched onto the heart muscle. Similar patches can be constructed for cells implanted into other organs, i.e., the brain or liver. Such an approach provides the advantage that the cells and formed tissues are readily retrievable following differentiation. Alternatively, some directed development may be performed *in vitro* in the presence of cells isolated from a mammal. Also, cells may be mixed with a matrigel substance, or other suitable extracellular matrix material which causes cells to aggregate, in order to encourage tissue formation, either for *in vivo* implantation or *in vitro* development in the presence of mixed cell types.

[0023] Preferably, the cells are inserted into a developing mammalian fetus that has not yet developed self recognition immune function. For example, a developing fetal sheep does not begin to develop self recognition until the age of 60 days (continuing to about 85 days), so it is possible to introduce human

cells before about day 55 to 60 and have the animal be tolerized to the implanted human cells. Thereafter, the human cells may differentiate without adverse immune response, even until the end of term, i.e., 145 days. Different points in time for implanting cells may be critical for different cell types and tissues. The criticality of timing is a parameter that may be readily analyzed by the skilled artisan given the present disclosure as a guide.

[0024] The invention also includes variations of the method discussed above as would be envisioned by the skilled artisan. For instance, antigens specific to the donor cells could be injected prior to the time period during which self recognition develops in the host fetus in order to tolerize the fetus to the foreign antigens. Then, cells to be encouraged along particular developmental pathways can be injected either during or after the development of self recognition without adverse immune response. This variation is particularly useful for encouraging differentiation into cell types found in organs that do not fully develop until after the period in which self recognition develops, i.e., thymus cells.

[0025] According to the methods of the invention, in order to further assist development along a certain path of differentiation, the embryonic pluripotent cells may be transfected with a selectable marker either prior to implantation, or prior to nuclear transfer, in order to help select cells which have differentiated along the proper pathway. The selectable marker may be any marker that may be employed in mammalian cells. For instance, the selectable marker may be selected from the group consisting of aminoglycoside phosphotransferase, puromycin, zeomycin, hygromycin, GLUT-2 and non-antibiotic resistance selectable marker systems. U.S. Patent No. 6,162, 433 discloses nonantibiotic selectable markers suitable for mammalian use, and is herein incorporated by reference in its entirety. A preferred selectable marker is aminoglycoside phosphotransferase, wherein said differentiated cells are selected by administering G418.

[0026] Although a developing fetal mammal is the preferred host environment for directing the development and differentiation of pluripotent cells, any mammalian host may be employed, i.e., including adults, embryos, fetuses and

embryoid bodies. For instance, the pluripotent cells of the invention may be implanted into an immune comprised host, such as a SCID mouse. In such an instance, the need to implant pre-tolerance is avoided, and implantation may be directed to more fully developed tissue locations. Also, the host environment need not necessarily be completely an *in vivo* environment. For instance, an embryoid body may be itself maintained *in vitro* depending on the stage of development sought and the extent of differentiation possible.

[0027] In systems where a selectable marker is employed, replacement cells and/or tissues may be purified by chemical selection *in vitro* or *in vivo*. A preferred method employs two separate selectable markers, for instance, neomycin and hygromycin, operably linked to promoters that are specifically expressed in an overlapping group of tissues. For instance, by expressing neomycin from a promoter specifically expressed in bone and splenocytes, and by also expressing hygromycin from a promoter specifically expressed in bone and cartilage, one can achieve a stronger selection for bone cells via an overlapping selection mechanism. In such a system, although the promoters are not cell-specific, the combined selection for both markers results in cell-specificity.

[0028] Alternatively, replacement cells may be purified away from surrounding host cells and tissues, for instance, using immunopurification targeting a cell-specific, species-specific cell surface protein. Antigens employed for purification of the desired cells may also be expressed via one or more exogenous gene construct(s) that are transfected into said primordial cells. Such exogenous genes may be preferentially expressed in the cells or tissues of interest via cell-specific or tissue –specific promoters. For example, the CD4 antigen can be preferentially expressed in pancreatic beta cells by operably linking the gene for the CD4 antigen to an insulin promoter. Because the CD4 antigen is not generally expressed in pancreatic, the seeded donor cells may be purified away from surrounding host cells by immunopurification using anti-CD4 antibodies, or via another purification process that targets the CD4 protein.

[0029] The present invention also encompasses the replacement cells and/or tissues produced by the methods disclosed herein, and methods of using the same to treat patients in need of replacement cells and tissues, i.e., via transplantation. The following examples serve to illustrate the disclosed invention, but should not be construed to limit the scope thereof.

EXAMPLE 1

[0030] A study is ongoing wherein primate, parthenogenetically derived cells (Cyno-1) are surgically implanted or injected into fetal lambs at different time points and different ages. Because the sheep does not develop self recognition until 60 to 85 days, there should be a difference in the stability and development of cells implanted prior to self recognition development and afterwards.

EXAMPLE 2

[0031] A study is underway to determine whether cell mixtures encourage differentiation of pluripotent cells either *in vivo* or *in vitro*. Briefly, Cyno-1 and other pluripotent cells will be mixed with fetal bud cells and placed under the kidney capsule of a SCID mouse in order to recover islet cells. Also, pluripotent cells may be mixed with pancreatic duct cells with or without Matrigel in order to aggregate cells into three dimensional structures, and examined both *in vivo* and *in vitro* for the development of islet cells.

EXAMPLE 3

Creation of Tissue-Engineered Renal Units by Nuclear Trasnfer

[0032] Tissue-engineered renal units were created from cells cloned from adult bovine fibroblasts. Urine-like fluid production and long-term viability were demonstrated after transplantation back into the nuclear donor animal despite expressing a different mtDNA haplotype. Examination of the explanted devices revealed formation of organized glomeruli and tubule-like structures. Immunohistochemical and RT-PCR analysis confirmed the expression of

specific renal mRNA and proteins exclusively in the cloned constructs, whereas the relative expression of CD4 and CD8 in allogeneic control units support the conclusion that there was no rejection response to the cloned cells following either primary or secondary (DTH test) challenge.

[0033] Specifically, we used nuclear transfer generate histocompatible renal structures in a large animal model, the cow (*Bos taurus*). Dermal fibroblasts isolated from an adult Holstein steer were used to clone a 56-day-old fetus as previous described (R.P. Lanza, *et al.*, *Science* **288**, 665 (2000).). Mitochondrial DNA (*mt*DNA) analysis confirmed a polymorphism between the cloned and nuclear donor cells at position 16127 (*Bos taurus* mitochondrial complete genome gi.5834939), demonstrating that although the clone and recipient were genetically identical at the nuclear level, they were distinguishable by their *mt*DNA haplotypes. Renal cells were isolated by enzymatic digestion using 0.1% collagenase/dispase, and passaged until the desired number of cells were obtained. In vitro immunocytochemistry confirmed expression of renal specific proteins, including synaptopodin (a protein produced by podocytes), aquaporin 1 (AQP1, a protein produced by proximal tubules and the descending limb of the loop of Henle), aquaporin 2 (AQP2, a protein produced by collecting ducts), Tamm-Horsfall protein (a protein produced by the ascending limb of the loop of Henle), and factor VIII (a protein produced by endothelial cells). Synaptopodin and AQP1 & 2 expressing cells exhibited circular and linear patterns in two-dimensional culture, respectively.

[0034] After expansion, the cells were trysinized, washed and seeded at a density of $2x10^7$/cells/cm^2 onto collagen-coated cylindrical polycarbonate membranes (3cm long, 10μm thick, 2μm pore size). Renal devices with collecting systems were constructed by connecting the ends of three membranes with 16 G Silastic catheters that terminated in a 2 ml reservoir made from polyethylene (Fig.1). Thirty-one units (n=19 with cloned cells, n=6 without cells, and n=6 with cells from an allogeneic, age-matched control fetus) were transplanted subcutaneously and retrieved 12 weeks after implantation back into the nuclear donor animal.

[0035] On gross examination, the explanted units appeared intact, and straw-yellow colored fluid could be observed in the reservoirs of the cloned group (Fig. 2A). There was a six-fold increase in volume in the experimental group vs the control groups (0.60±0.04 ml vs 0.10±0.01 ml and 0.13±0.04 ml in the allogeneic and unseeded control groups, respectively, P<0.00001). Chemical analysis of the fluid suggested unidirectional secretion and concentration of urea nitrogen (18.3±1.8 mg/dl urea nitrogen in the cloned group vs 5.6±0.3 mg/dl and 5.0±0.01 mg/dl in the allogeneic and unseeded control groups, respectively, P<0.0005)(the blood urea nitrogen level was 8.3±0.3 mg/dl) and creatinine (2.5±0.18 mg/dl creatinine in the cloned group vs 0.4±0.18 mg/dl and 0.4±0.08 mg/dl in the allogeneic and unseeded control groups, respectively, P<0.0005)(the blood creatinine level was 0.7±0.16 mg/dl).

[0036] The retrieved implants demonstrated extensive vascularization, and glomeruli and tubule-like structures. The later were lined with cuboid epithelial cells with large, spherical and pale-stained nuclei (Fig 3A), whereas the glomeruli-like structures exhibited a variety of cell types with abundant red blood cells (Fig. 3B). Immunohistochemical analysis confirmed expression of renal specific proteins, including AQP1, AQP2, synaptopodin, and factor VIII (Fig. 4). Antibodies for AQP1, AQP2, and synaptopdin identified tubular, collecting tubule, and glomerular segments within the constructs, respectively. In contrast, the allogeneic controls displayed a foreign body reaction with necrosis, consistent with the finding of acute rejection. RT-PCR analysis confirmed the transcription of AQP1, AQP2, synaptopodin, and Tamm-Horsfall genes exclusively in the cloned group (Fig. 4). Cultured and cloned cells also expressed high protein levels of AQP1, AQP2, synaptopodin, and Tamm-Horsfall protein as determined by Western blot analysis. Expression intensity of CD4 and CD8, markers for inflammation and rejection, were also significantly higher in the control vs cloned group (Fig.4).

[0037] Although the cloned cells derived their nuclear genome from the original fibroblast donor, their *mt*DNA was derived from the original recipient oocyte. Therefore, the oocyte *mt*DNA genetically distinguished the cloned cells from the nuclear donor and ultimate recipient of the renal transplants. A

relatively limited number of *mt*DNA polymorphisms have been shown to define what have been referred to as maternally transmitted minor histocompatibility antigens (miHA) in mice (K. Fischer Lindahl, E. Hermel, B.E. Loveland, & C.R.Wang, *Ann.Rev. Immunol,* **9**, 351 (1991).). This class of miHA has been shown to stimulate both skin allograft rejection in vivo and expansion of cytotoxic T lymphocytes (CTL) in vitro (only after in vivo priming) (K. Fischer Lindahl, E. Hermel, B.E. Loveland, & C.R.Wang, *Ann.Rev. Immunol,* **9**, 351 (1991).). It was important to investigate the potential T lymphocyte response of the renal transplant recipient to the cloned renal devices. Although the contribution of *mt*DNA-coded genes to immunocompatibility is likely to be minimal, chronic T lymphocyte responses to miHA could constitute a barrier to successful clinical use of such cloned devices as hypothesized for chronic rejection of MHC-matched human renal transplants (G.A. Hadley, B. Linders, & T. Mohanakumar, *Transplantation* **54**, 537 (1992) and B.A.Yard, *et al., Kidney Intl.* **43**, S133 (1993).).

[0038] We chose to investigate a possible anti-miHA T cell response to the cloned renal devices through delayed-type hypersensitivity (DTH) testing. An in vivo assay of anti-miHA immunity was chosen based on (R.P.Lanza, J.B. Cibelli, & M.D.West, *Nat. Biotechnol.* **17**, 1171 (1999).) the ability skin allograft rejection to detect a wide range of miHA in mice with survival times exceeding 10 weeks (Bailey, D.W. *Immunogenetics* **2**, 249 (1975).) and (Data from: *UNOS Critical Data: U.S. Facts about Transplantation* (February 1, 2002), available at http://www.unos.org/Newsroom/critdata_main.htm) the relative insensitivity of in vitro assays in detecting miHA incompatibility, highlighted by the requirement for in vivo priming to generate CTL (T. Mohanakumar, *The role of MHC and non-MHC antigens in allograft immunity* (R.G. Landes Company, Austin, Texas, 1994) pp. 1-115.). We were unable to discern an immunological response directed against the cloned cells by DTH testing *in vivo*. Cloned and control allogeneic cells were intra-dermally injected back into the nuclear donor animal (1×10^6 cells in 0.1 ml in triplicate sites) 80 days after the initial transplantation. A positive DTH response was observed after 48 hours for the allogeneic control cells but not the cloned cells (diameter of

erythema/induration approx 9 x 4.5 mm, 12 x 10 mm, and 11 x 11 mm vs 0, 0, and 0 mm, respectively, P<0.02). These results corroborate the relative CD4 and CD8 expression in Western blots (Fig. 4) and support the conclusion that there was no detectable rejection response to cloned cells following either primary or secondary challenge.

[0039] Previous efforts at tissue-engineering the kidney have been directed toward development of an extracorporeal renal support system comprising both biologic and synthetic hemofiltration components (H.D. Humes, D.A. Buffington, S.M. MacKay, A.J. Funke & W.F. Weitzel, *Nat. Biotechnol.* 17, 451 (1999).; D.A. Cieslinski & H.D. Humes, *Biotechnol. Bioeng.* 43, 781 (1994).; and S.M. MacKay, A.J. Kunke, D.A. Buffington & H. D. Humes *ASAIO J.* 44, 179 (1998).). Although such ex vivo organ substitution therapy would be life-sustaining, there would be obvious benefits for patients if such devices could be implanted long-term without the need for an extracorporeal perfusion circuit or immunosuppressive drugs and/or immunomodulatory protocols. Our results suggest that it may be feasible to use therapeutic cloning to generate functional renal tissues. Cloned renal cells were successfully expanded in vitro, seeded onto renal units, and grafted back into the nuclear donor organism without immune destruction despite having allogeneic *mt*DNA. The cells organized into glomeruli- and tubule-like structures with the ability to excrete toxic metabolic waste products through a urine-like fluid.

[0040] Although stem-like cells have been produced by nuclear transfer in cattle (15), little success has been achieved in inducing them to differentiate into specified tissue in vitro. Therefore, it was necessary in the present study to generate an early-stage fetus. In contrast, human and primate embryonic stem (ES) cells have been successfully differentiated in vitro into derivatives of all three germ layers (16-20). In humans, there is an ethical consensus not to allow pre-implantation embryos to develop in vitro beyond the blastocyst stage; but rather to derive primordial stem cells from the inner cell mass as a source of genetically matched cells for transplantation (1,21). The ability to bioengineer these cells into more complex functional structures such as kidneys, livers, or

even entire hearts (22, 23), would overcome the two major problems in transplantation medicine: immune rejection and organ shortage.

EXAMPLE 4

Creation of Tissue-Engineered Cardiac and Skeletal Tissues By Nuclear Transfer in Bos Taurus

[0041] In the present study, we tested the histocompatibility of nuclear-transfer-generated cells and tissues in a large animal model, the cow (*Bos taurus*). Cloned cardiac and skeletal muscle cell implants were not rejected, and they remained viable after being transplanted back into the nuclear donor animal. In addition to demonstrating long-term viability of second-set grafts, RT-PCR and Western blot analyses confirmed the expression of specific mRNA and proteins in the retrieved explants despite expressing a different mtDNA haplotype.

Results and discussion

[0042] **Histochemical and immunocytochemical analysis.** Tissue engineered constructs containing bovine cardiac (n=8) and skeletal muscle cells (n=8) were transplanted subcutaneously and retrieved 6 weeks after implantation. After retrieval of the first-set implants, a second set of constructs containing cardiac (n=6) and skeletal muscle cells (n=6) from the same donor were transplanted for a further 12 weeks. On a histological level, the cloned cardiac tissue appeared intact, and showed a well-organized cellular orientation with spindle-shaped nuclei (Fig. 5A). The retrieved tissue stained positively with troponin I antibodies, indicating the preservation of cardiac muscle phenotype (Fig.5B). The cloned skeletal muscle cell explants showed spatially oriented tissue bundles with elongated multinuclear muscle fibers (Fig.5D,G). Immunohistochemical analysis using sarcomeric tropomyosin antibodies identified skeletal muscle fibers within the implanted constructs (Fig.5F). In contrast to the cloned implants, the allogeneic, control cell implants failed to form muscle bundles, and showed an increased number of inflammatory cells, fibrosis, and necrotic debris consistent with acute rejection (Fig. 5H,I).

[0043] Histological examination revealed extensive vascularization throughout the implants, as well as the presence of multinucleated giant cells surrounding the remaining polymer (polyglycolic acid)(PGA) fibers. Although non-degraded polymer fibers were present in all tissue specimens, histomorphometric analysis of the explanted tissues indicated that the degree of immune reaction was significantly less in the cloned versus control tissue sections (66±4 and 54±4 [mean±SEM] total inflammatory cells/HPF/cloned constructs at 6 weeks [first-set grafts] and 12 weeks [second-set grafts], respectively, vs. 93±3 and 80±3 cells/HPF for the constructs generated from the control cells, $P<0.0005$; and 33±3 and 29±3 lymphocytes/HPF/cloned constructs at 6 and 12 weeks respectively, vs. 52±4 and 46±3 cells/HPF for the constructs generated from the control cells; $P<0.005$) (Fig. 5F-G). Immunocytochemical analysis using CD4- and CD8-specific antibodies identified an approximately twofold increase in CD4+ and CD8+ T cells (13±1.3 and 14±1.4 cells/HPF, respectively, vs. 7±1.1 and 7±1.2 cells/HPF, $P<0.00001$) within the explanted first and second set control vs. cloned constructs. Importantly, first and second set cloned constructs exhibited comparable levels of CD4 and CD8 expression, arguing against the presence of an enhanced second set reaction as would be expected if mtDNA-encoded minor antigen differences were present.

[0044] Poly(glycolic acid) is one of the most widely used synthetic polymers in tissue engineering (11). PGA polymers are attractive due to their biodegradability and biocompatibility, and have been used in experimental and clinical settings for decades as scaffolds for delivering cells and drugs, absorbable suture materials and prosthetic implants. Although the scaffolds are immune acceptable, the PGA construct is known to stimulate a characteristic pattern of inflammation and fibrovascular ingrowth similar to that observed in the cloned constructs of the present study. However, this response, which is greatest at around 12 weeks of implantation, can be considered separate from the immune response to the transplanted cells, even though there obviously can be interactions between the two (Santavirta, S., Konttinen, Y.T., Saito, T., Gronblad, M., Partio, E., Kemppinen, P. & Rokkanen, P. Immune response to

polyglycolic acid implants. *J.Bone Joint Surg. Br.* **72**, 597-600 (1990).; Paivarinta, U., Bostman, O., Majola, A., Toivonen, T., Tormala, P. & Rokkanen, P. Intraosseous cellular response to biodegradable fracture fixation screws made of polyglycolide or polylactide. *Arch. Orthop. Trauma Surg.* **112**, 71-74 (1993).; Bostman, O.M. & Pihlajamaki, H.K. Adverse tissue reactions to bioabsorbable fixation devices. *Clin.Orthop.* **371**, 216-27 (2000); Ruuskanen, M., Ashammakhi, N., Kallioinen, M., Pohjonen, T., Tormala, P. & Waris, T. Evaluation of self-reinforced polyglycolide membrane implanted in the subcutis of rabbits. *Ann. Chir. Gynaecol.* **88**, 308-12 (1999).; and Weiler, A., Helling, H.J., Kirch, U., Zirbes, T.K. & Rehm, K.E. Foreign-body fracture fixation: experimental study in sheep. *J. Bone Joint Surg. Br.* **78**, 369-76 (1996).).

[0045] **RT-PCR and Western blot analyses.** Semi-quantitative RT-PCR and Western blot analysis confirmed the expression of specific mRNA and proteins in the retrieved tissues despite the presence of allogeneic mitochondria. Mean expression intensities of myosin/GAPDH and troponin T/GAPDH in the cloned skeletal and cardiac muscle implants were 0.22±0.03 and 0.15±0.02 (6 weeks) and 0.09±0.08 and 0.29±0.1 (12 weeks), respectively. In contrast, expression intensities were significantly lower or absent in constructs generated from genetically unrelated cattle (0.02±0.01 and 0±0.00 at 6 weeks, $P<0.005$; and 0±0.01 and 0.02±0.1 at 12 weeks, $P<0.05$)(Fig. 6 and Table 1). The cardiac and skeletal muscle explants also expressed high protein levels of desmin and troponin I as determined by Western blot analysis (Fig. 7 and Table 2). Desmin expression was significantly greater in the cloned versus control tissue sections (85±1 and 68±4 vs. 30±2 and 16±2 at 6 weeks for the skeletal and cardiac implants, respectively, $P<0.001$; and 80±3 and 121±24 vs. 53±2 and 52±8 at 12 weeks for the constructs generated from the skeletal and cardiac cells, $P<0.05$). The expression intensities of troponin I in the cloned and control cardiac muscle explants was 68±4 and 16±2 at 6 weeks ($P<0.001$), respectively, and 94±7 and 54±12 at 12 weeks ($P<0.05$).

[0046] Western blot analysis of the first-set explants indicated an approximately six-fold increase in expression intensity of CD4 in the control versus cloned constructs at 6 weeks (30±10 and 32±3 for the control skeletal

and cardiac implants, respectively, vs. 5±1 and 5±1 for the cloned skeletal and cardiac constructs)($P<0.0005$), confirming a primary immune response to the control grafts. There was also a significant increase in the mean expression intensities of CD8 in the control versus cloned constructs at 6 weeks (26±5 vs. 15±4, $P<0.05$). Twelve weeks after second-set implantation, mean expression intensities of CD4 and CD8 continued to remain significantly elevated in the control vs cloned constructs (23±4 vs. 12±3 for CD4, respectively, and 54±7 vs. 26±2 for CD8, $P<0.005$).

[0047] Mitochondrial DNA (*mt*DNA) analysis. Previous studies showed that bovine clones harbor the oocyte mtDNA (Evans, M.J., Gurer, C., Loike, J.D., Wilmut, I., Schnieke, A.E. & Schon, E.A. *Nat. Genet.* **23**, 90-93 (1999).; Hiendleder, S., Schmutz, S.M., Erhardt, G., Green, R.D. & Plante, Y. Mol. *Reprod. Dev.* **54**, 24-31 (1999). ; Steinborn, R. et al. Mitochondrial DNA heteroplasmy in cloned cattle produced by fetal and adult cell cloning. *Nat. Genet.* **25**, 255-257 (2000). and Lanza, R.P. et al. Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. *Cloning* **2**, 79-90 (2000).). As discussed above, differences in mtDNA-encoded proteins expressed by clone cells could stimulate a T cell response specific for mtDNA-encoded minor histocompatibility antigens (Lanza, R.P. et al. Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. *Cloning* **2**, 79-90 (2000).) when clone cells are transplanted back to the original nuclear donor. The most straight-forward approach to resolve the question of minor antigen involvement is the identification of potential antigens by nucleotide sequencing of the mtDNA genomes of the clone and fibroblast nuclear donor. The contiguous segments of mtDNA that encode 13 mitochondrial proteins and tRNA's were amplified by PCR from total cell DNA in five overlapping segments. These amplicons were directly sequenced on one strand with a panel of sequencing primers spaced at ≈500 bp intervals.

[0048] The resulting nucleotide sequences (13,210 bp) revealed only nine nucleotide substitutions (Table 3). One substitution was in the tRNA-Gly segment and five substitutions were synonymous. The sixth substitution, in the ND1 gene, was heteroplasmic in the nuclear donor (transplant recipient) where

one of the two alternative nucleotides was shared with the clone. A Leu or Arg would be translated at this position in ND1. The eighth and ninth substitutions resulted in amino acid interchanges of Asn > Ser and Val > Ala in the ATPase6 and ND4L genes, respectively. The identification of two amino acid substitutions that distinguish the clone and the nuclear donor confirm that a maximum of only two minor histocompatibility antigen peptides could be defined by this donor:recipient transplant combination. Given the lack of knowledge concerning peptide binding motifs for bovine MHC class I molecules, there is no reliable method to predict the impact of these amino acid substitutions on the ability of mtDNA-encoded peptides to either bind to bovine class I molecules or activate CD8+ CTLs.

[0049] Despite the potential involvement of this minimal number of amino acid substitutions, it was clear that first- and second-set clone devices functionally survived for the duration of these experiments without significant increases in infiltration of second-set devices by CD4+ and CD8+ T lymphocytes. Specifically, cloned cardiac and skeletal tissues remained viable >3 months after second-set transplantation (comparable to in vitro control specimens). Multiple, viable, myosin- and troponin I-containing cells were observed throughout the tissue constructs, consistent with functionally active protein synthesis and expression. This direct and relevant assessment of graft function does not provide any evidence to support the activation of a T cell response to cloned tissue-specific histocompatibility antigens in this donor:recipient combination. Supporting evidence for a lack of response to minor histocompatibility antigens expressed by transplanted, cloned tissues comes from preliminary experiments with a comparable transplant recipient where peripheral blood lymphocytes harvested after transplant harvest did not proliferate in vitro when stimulated with cloned cells in mixed lymphocyte culture but did proliferate in response to allogeneic cells (data not shown).

[0050] Our results suggest that cloned cells and tissues can be grafted back into the nuclear donor organism without immune destruction despite having allogeneic *mt*DNA, although further studies will be necessary to rule out the possibility of immune rejection with other donor recipient transplant

combinations. It is also important to note that while stem-like cells have been produced by nuclear transfer in cattle (Cibelli, J.B. et al. Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. *Nat. Biotech.* **16**, 642-646 (1998).), little success has been achieved in inducing them to differentiate into specified tissue in vitro. Therefore, it was necessary in the present study to generate an early-stage bovine embryo. In contrast, human and primate embryonic stem (ES) cells have been successfully differentiated in vitro into derivatives of all three germ layers, including beating cardiac muscle cells, smooth muscle, and insulin- and dopamine-producing cells, among others (Itskovitz-Eldor, J et al. Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. *Mol. Med* **5**, 88-95 (2000).; Schuldiner, M. Yanuka, O., Itskovitz-Eldor, J, Melton, D.A. & Benvenisty, N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. Natl. Acad. Sci USA* **97**, 11307-11312 (2000).; Kaufman, D.S. et al. Directed differentiation of human embryonic stem cells into hematopoeitic colony forming cells. *Blood* **94** (suppl 1, part 1 of 2), 34a (1999).; Reubinoff, B.E. et al. Neural progenitors from human embryonic stem cells. *Nat. Biotech.* **19**, 1134-1140 (2001).;Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotech.* **18**, 399-404 (2000). and Cibelli, J.B. et al. Parthenogentic stem cells in nonhuman primates. *Science* (in press).). In humans, development would not be taken beyond the blastocyst stage; rather, the goal is to derive primordial stem cells in vitro, such as ES cells, as a source of cells for tissue engineering and regenerative medicine (Lanza, R.P., Cibelli, J.B. & West, M.D. Human therapeutic cloning. *Nat. Med.* **5**, 975-977 (1999).; Cibelli, J.B., Kiessling, A.A., Cunniff, K., Richards, C., Lanza, R.P. & West, M.D. Somatic cell nuclear transfer in humans: pronuclear and early embryonic development. *e-biomed: J. Regen. Med.* **2**, 25-31 (2001).; andLanza, R.P. et al. The ethical validity of using nuclear transfer in human transplantation. *J.A.M.A.* **284**, 3175-3179 (2000).).

Experimental protocol

[0051] Mitochondrial DNA analyses. Mitochondrial DNA products ranging in size from 3 to 3.8kb were amplified by PCRs using Advantage-GC Genomic Polymerase (Clontech, Palo Alto, CA) and total genomic DNA templates from the clone and nuclear donor. The regions of the mitochondria that were amplified included all of the protein-coding sequences and the intervening tRNAs. PCR products were electrophoresed in 1% SeaPlaque GTG agarose (Rockland, ME), extracted from the gels with the use of QIAquick Gel Extraction Kits (Qiagen, Valencia, CA), and sequenced by the Molecular Biology Core Facility (Mayo Clinic, Rochester, MN) with a series of primers located approximately 500 base intervals. Sequences have been submitted to Genbank with Bankit #'s 456319 and 456325.

[0052] Adult bovine cell line derivation. Dermal fibroblasts were isolated from an adult Holstein steer. To obtain fibroblasts for nuclear transfer, a skin biopsy was obtained from the animal by ear notch. The tissue sample was minced and cultured in DMEM supplemented with 15% fetal calf serum, L-glutamine (2 mM), non-essential amino acids (100 μM), β mercaptoethanol (154 μM) and antibiotics at 38°C in a humidified atmosphere of 5% CO_2 and 95% air. The tissue explants were maintained in culture and a fibroblast cell monolayer established. The cell strain was maintained in culture, passaged twice and cryopreserved in 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Donor cells were thawed, cultured, passaged and further propagated prior to nuclear transfer.

[0053] Nuclear transfer and embryo culture. Bovine *(Bos taurus)* oocytes were obtained from abattoir-derived ovaries (from multiple animals) as previously described (Lanza, R.P. et al. Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. *Cloning* 2, 79-90 (2000).). Oocytes were mechanically enucleated at 18-22 hours post maturation, and complete enucleation of the metaphase plate confirmed with *bis*Benzimide (Hoechst 33342) dye under fluorescence microscopy. A suspension of actively dividing cells was prepared immediately prior to nuclear transfer. The cell suspension was centrifuged at 800 x g and 5 μl of the resulting cell pellet used for the donor

cells. A single cell was selected and transferred into the perivitelline space of the enucleated oocyte. Fusion of the cell-oocyte complexes was accomplished by applying a single pulse of 2.4 kV/cm for 15 μsec. Nuclear transfer embryos were activated as previously described by Presicce et al (Presicce, G.A. & Yang, X. Parthenogenetic Development of Bovine Oocytes Matured In Vitro for 24 Hr and Activated by Ethanol and Cycloheximide. *Molecular Reproduction and Development* **38**, 380-385 (1994)) with slight modifications. Briefly, reconstructed embryos were exposed to 5 μM of Ionomycin (Calbiochem) in TL Hepes (Blowhittaker) for 5 minutes at room temperature followed by a 6 hours incubation with 5 μg/ml of Cytochalasin B (Sigma) and 10 μg/ml of Cycloheximide (Sigma) in ACM media at 38.5 C and 5% CO2 in air. Cleavage rates were recorded and development to the blastocyst stage was assessed on days 7 and 8 of culture. Resulting blastocysts were non-surgically transferred into progestrin-synchronized recipients.

[0054] Cell culture and seeding. Cardiac muscle and skeletal muscle tissue from 5 to 6 week-old cloned and natural (control) fetuses were retrieved. Cardiac and skeletal cells were isolated by the explant technique using DMEM supplemented with either 15% heat-inactivated fetal calf serum (FCS) (HyClone Laboratories, Inc., Logan, Utah), plus the following: 1% penicillin-streptomycin, 1% L-glutamine, 1% nonessential amino acids, and 1% β-mercaptoethanol. The cells were incubated in a humidified atmosphere chamber containing 5% CO_2 and maintained at 37°C. Cells were passed by 1:3 or 1:4 every 3 to 4 days. Both muscle cell types were expanded separately until desired cell numbers were obtained. The cells were trypsinized, collected, washed and counted for seeding. Cells were seeded in 1 x 2 cm PGA polymer scaffolds with $5x10^7$ cells. Vials of frozen donor cells were thawed, cultured, passaged and further propagated prior to seeding the second-set scaffolds.

[0055] Polymers. Unwoven sheets of polyglycolic acid polymers (1cm x 2cm x 3mm) were used as cell delivery vehicles (Albany International, Mansfield, MA). The polymer meshes were composed of fibers of 15μm in diameter and an interfiber distance between 0 - 200 um with 95% porosity. The scaffold was designed to degrade via hydrolysis in 8-12 weeks. The polymers were

sterilized in ethylene oxide and placed under sterile conditions until cell delivery.

[0056] Implantation. The cell-polymer constructs were implanted into the flank subcutaneous tissue of the same steer from which the cells were cloned; skeletal muscle and cardiac muscle constructs derived from a single fetus (and thus a single oocyte) were used for implantation. Fourteen constructs (8 first-set and 6 second-set) for each cell type were implanted. Control group constructs, with cells isolated from a single allogeneic fetus, were implanted on the contralateral side. The implanted constructs were retrieved at 6 weeks (first-set) and 12 weeks (second-set) after implantation.

[0057] Histological and immunohistochemical analyses. Five-micron sections of 10% buffered formalin fixed paraffin-embedded tissue were cut and stained with hematoxylin and eosin (H&E). Immunohistochemical analyses were performed using specific antibodies in order to identify the cell types in retrieved tissues with cryostat and paraffin sections. Monoclonal sarcomeric tropomyosin (Sigma, St. Louis, MO) and troponin I (Chemicon, Temecula, CA) antibodies were used to detect skeletal and cardiac muscle fibers, respectively. Monoclonal CD4 and CD8 (Serotec, Raleigh, NC) antibodies were used to identify T cells for immune rejection. Specimens were routinely processed for immunostaining. Pretreatment for high–temperature antigen unmasking pretreatment with 0.1% trypsin was performed using a commercially available kit according to the manufacturer's recommendations (T–8128; Sigma). Antigen–specific primary antibodies were applied to the deparaffinized and hydrated tissue sections. Negative controls were treated with non-immune serum instead of the primary antibody. Positive controls consisted of normal cardiac and skeletal tissue. After washing with phosphate buffered saline, the tissue sections were incubated with a biotinylated secondary antibody and washed again. A peroxidase reagent (DAB) was added. Upon substrate addition, the sites of antibody deposition were visualized by a brown precipitate. Counterstaining was performed with Gill's hematoxylin. For determining the degree of immunoreaction, the immune cells were counted

under 5 high power fields per section (HPF, x200) using computerized histomorphometrics (BioImaging Analyses Software).

[0058] RNA isolation, cDNA synthesis. Fresh retrieved tissue implants were harvested and frozen immediately in liquid nitrogen. The tissue was homogenized in RNAzol reagent at 4°C using a tissue homogenizer. RNA was isolated according to the manufacturers protocol (Tel-Test). Complementary DNA was synthesized from 2 ug RNA using the SuperscriptII reverse transcriptase (GIBCO) and random hexamers as primers.

[0059] PCR. For PCR amplification 1 ml of cDNA with 1 U Taq DNA polymerase (Roche), 200mM dNTP and 10 pM of each primer were used in a final volume of 30 ml. Myosin for skeletal muscle tissue was amplified from cDNA with primers 5'-TGAATTCAAGGAGGCGTTTCT-3' and 5'-CAGGGCTTCCACTTCTTCTTC-3'. Troponin T for cardiac tissue was done with primer 5'-AAGCGCATGGAGAAGGACCTC-3' and 5'-GGATGTAGCCGCCGAAGTG-3'. PCR products were visualized with agarose gel electrophoresis and ethidium bromide staining.

[0060] Western blot analysis. Implanted tissue was harvested, stored in liquid nitrogen and homogenized in lysis buffer using a tissue homogenizer. After measuring protein concentration (BioRad), equal protein amounts were loaded on 10% SDS-PAGE. Proteins were blotted onto PVDF-membranes, the membranes were incubated with primary antibodies for 1 hour at room temperature. Desmin and actin (Santa Cruz Biotech, Santa Cruz, CA) antibodies were used to detect skeletal muscle tissue, and desmin (Santa Cruz Biotech, Santa Cruz, CA) and troponin I (Chemicon, Temecula, CA) antibodies were used to detect cardiac muscle tissue. Monoclonal CD4 and CD8 (Serotec, Raleigh, NC) antibodies were used as markers for inflammation and rejection. Subsequently membranes were incubated with secondary antibodies for 30 minutes. The signal was visualized using the ECL system (NEN, Boston, MA).

[0061] Statistical analysis. Data are presented as mean ±SEM and compared using the two-tailed Student's *t* test. Differences were considered significant at $P<0.05$.

Table 1. RT-PCR analysis of tissues

	Control 6wks	Cloned 6wks	Control 12wks	Cloned 12wks
Skeletal (myosin/GAPDH)	0.02±0.01*	0.22±0.03*	0±0.01**	0.09±0.04**
Cardiac (troponin T/GAPDH)	0±0.00***	0.15±.02***	0±0.02**	0.29±0.05**

Mean±SEM

* P<0.005

** P<0.05

*** P<0.001

Table 2. Western blot analysis of tissues

		Control 6wks	Cloned 6wks	Control 12wks	Cloned 12wks
Skeletal	Desmin	30±2*	85±1*	53±2**	80±3**
Cardiac	Troponin I	23±6***	75±1***	54±12****	94±7****
	Desmin	16±2***	68±4***	52±8****	121±24****

Mean ±SEM

* P<0.0001

** P<0.005

*** P<0.001

**** P<0.05

Table 3. Nucleotide and amino acid substitutions that distinguish the nuclear donor and cloned cells

Clone	Nuclear Donor	Position [a]	Gene	Amino Acid Substitution	Surrounding Peptide Sequence
A	G	13080	ND5	-	
T	C	14375	ND6	-	
T	C	7851	Coll	-	
C	T	8346	ATPase6	-	
A	G	8465	ATPase6	N>S	QLVSKMMNIHNSKGQT
	S				
G	G/T	3501	ND1	R→ L/R	
C	T	9780	tRNA-Gly		
T	C	10,432	ND4L	V→A	SMMPIILLVFAACEAAL
					A
G	A	11,476	ND4	-	

[a]Position in Genbank #J013494

What is claimed:

1. A method of producing replacement cells and/or tissues for a mammal in need of such replacement cells and/or tissues, comprising:

(a) isolating a primordial stem cell or other embryonic pluripotent cell or cells;

(b) introducing into said primordial stem cell or embryonic pluripotent cell(s) at least one selectable marker operatively linked to a cell or tissue specific promoter, enhancer or other regulatory genetic element such that said selectable marker is expressed in the cell or tissue type of interest;

(c) permitting said primordial stem cell or embryonic cell(s) to differentiate into differentiated cells and tissues; and

(d) selecting for cells and tissues that express said selectable marker in order to produce replacement cells and/or tissues.

2. The method of claim 1, wherein said mammal is a human.

3. The method of claim 1, wherein said replacement cells and/or tissues are selected from the group consisting of pancreatic beta cells, brain cells, neurons, cardiomyocytes, fibroblasts, skin cells, liver cells, kidney cells, muscle cells, nerve stem cells, hematopoietic cells.

4. The method of claim 1, wherein said primordial stem cell or embryonic pluripotent cell(s) are selected from the group consisting of embryos having from about one to sixty-four cells, ES cells and ICM cells.

5. The method of claim 4, wherein said primordial stem cell or embryonic pluripotent cell(s) are isolated from naturally-, IVF- or nuclear-transfer generated embryos.

6. The method of claim 1, wherein said selectable marker is selected from the group consisting of aminoglycoside phosphotransferase, puromycin, zeomycin, hygromycin, GLUT-2 and non-antibiotic resistance selectable marker systems.

7. The method of claim 6, wherein said selectable marker is aminoglycoside phosphotransferase, and said differentiated cells are selected by administering G418.

8. The method of claim 1 wherein step (3) of permitting said primordial stem cell or embryonic pluripotent cells to differentiate is performed *in vivo* by injecting or implanting said primordial stem cell or embryonic pluripotent cells into a host animal selected from the group consisting of an adult, embryo, fetus or embryoid body.

9. The method of claim 8, wherein said adult animal is a SCID or nude mouse.

10. The method of claim 8, wherein the primordial stem cell or embryonic pluripotent cells are implanted into an intrauterine fetus.

11. The method of claim 8, wherein said embryoid body is itself maintained *in vitro*.

12. The method of claim 8 further comprising a step (5) wherein said resultant replacement cells and/or tissues are purified away from the surrounding host cells and tissues.

13. The method of claim 12, wherein said replacement cells and/or tissues are purified by chemical selection *in vitro*.

14. The method of claim 12, wherein said replacement cells and or tissues are purified by virtue of surface proteins.

15. The method of claim 1, wherein said primordial stem cell or embryonic pluripotent stem cells are genetically engineered by inserting, deleting or modifying a gene or other genetic material in addition to said selectable marker prior to differentiation.

16. The replacement cells and/or tissues produced by the method of claim 1.

17. A method of treating a patient in need of such replacement cells and/or tissues by transplanting into said patient the replacement cells and/or tissues produced by the method of claim 1.

18. A method of producing replacement cells and/or tissues for a mammal in need of such replacement cells and/or tissues, comprising:

(a) isolating a primordial stem cell or embryonic pluripotent cell or cells;

(b) injecting or implanting said primordial stem cell or embryonic pluripotent cell(s) into a host animal;

(c) permitting said primordial stem cell or embryonic cell(s) to differentiate into differentiated cells and tissues; and

(d) isolating said differentiated cells and tissues in order to produce replacement cells and/or tissues.

19. The method of claim 18, wherein said mammal is a human.

20. The method of claim 18, wherein said embryonic pluripotent cell(s) are selected from the group consisting of embryos having from about one to sixty-four cells, ES cells and ICM cells.

21. The method of claim 18, wherein said primordial stem cell or embryonic pluripotent cell(s) are isolated from naturally-, IVF- or nuclear-transfer generated embryos.

22. The method of claim 18, wherein said replacement cells and or tissues are isolated by virtue of surface proteins.

23. The method of claim 18 wherein said host animal is selected from the group consisting of an adult, embryo, fetus or embryoid body.

24. The method of claim 23, wherein said adult host animal is a SCID or nude mouse.

25. The method of claim 23, wherein said primordial stem cell or embryonic pluripotent cell(s) are implanted into an intrauterine fetus.

26. The method of claim 23, wherein said host embryoid body is itself maintained *in vitro*.

27. The method of claim 18, wherein said primordial stem cell or embryonic pluripotent cell(s) are genetically engineered by inserting, deleting or modifying a gene or other genetic material prior to injecting or implanting said cells into a host animal.

28. The method of claim 27, wherein a selectable marker is inserted into said primordial stem cell or embryonic pluripotent cell(s) prior to injecting or implanting said cells into a host animal.

29. The method of claim 28, wherein said selectable marker is selected from the group consisting of aminoglycoside phosphotransferase, puromycin, zeomycin, hygromycin, GLUT-2 and non-antibiotic resistance selectable marker systems.

30. The method of claim 29, wherein said selectable marker is aminoglycoside phosphotransferase, and said differentiated cells are selected *in vivo* by administering G418 to said host animal.

31. The method of claim 29, wherein said selectable marker is aminoglycoside phosphotransferase, and said differentiated cells are selected *in vitro* by administering G418 to said differentiated cells and tissues after they are isolated from said host animal.

32. The method of claim 23, wherein said host animal is an adult or fetus, and said embryonic pluripotent cell(s) are injected or implanted into the brain, pancreas, liver, kidney, kidney capsule, pancreatic duct or heart muscle wall of said host animal.

33. The method of claim 32, wherein said replacement cells and/or tissues are selected from the group consisting of cardiomyocytes, pancreatic beta cells, brain cells, neurons, liver cells, kidney cells and muscle cells.

34. The method of claim 23, wherein said host animal is an embryo or embryoid body, and said embryonic pluripotent cell(s) are injected or implanted into the heart region, the ectodermal region, mesodermal region, endodermal region or beneath the kidney capsule.

35. The method of claim 34, wherein said replacement cells and/or tissues are selected from the group consisting of cardiomyocytes, pancreatic beta cells, brain cells, neurons, liver cells, kidney cells and muscle cells.

36. The replacement cells and/or tissues produced by the method of claim 18.

37. A method of treating a patient in need of such replacement cells and/or tissues by transplanting into said patient the replacement cells and/or tissues produced by the method of claim 18.

38. The method of claim 1, wherein at least two different selectable marker constructs are introduced into said primordial stem cell or embryonic pluripotent cell(s).

39. The method of claim 39, wherein said at least two different selectable marker constructs are expressed in overlapping groups of tissues or cell types.

40. The method of claim 1, wherein said primordial stem cell or embryonic pluripotent cell(s) are transfected with at least one exogenous gene expressing a cell surface protein operably linked to a cell-specific or tissue specific promoter, whereby expression of said exogenous gene allows for purification of said transfected cells.

41. The method of claim 40, wherein said at least one exogenous gene encodes a CD4 antigen.

42. The method of claim 40, wherein said at least one exogenous gene is operably linked to an insulin promoter.

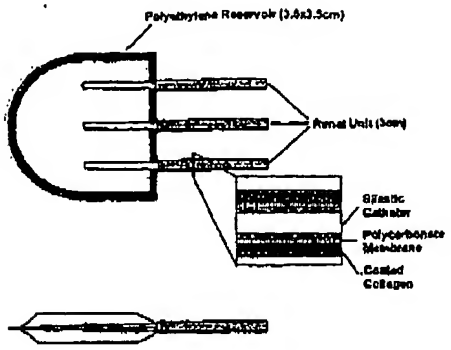
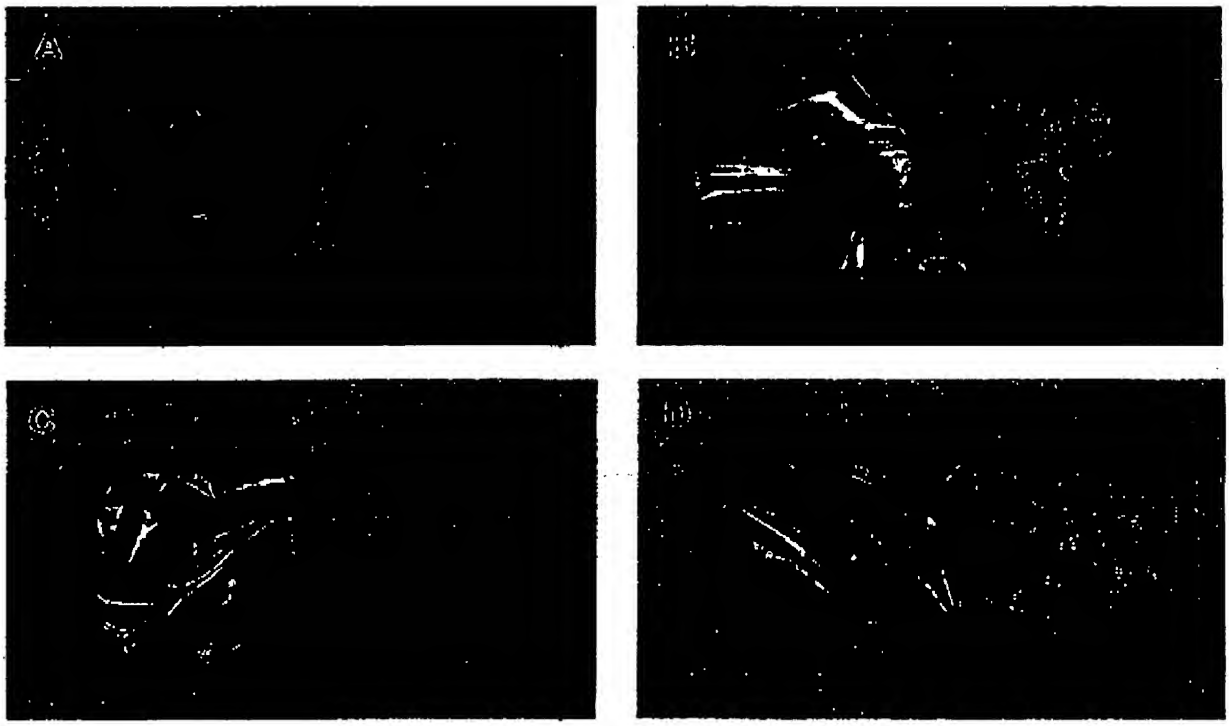

Polyethylene Reservoir (3.6x3.5cm)
Sintered Unit (3cm)
Silastic Catheter
Polycarbonate Membrane
Coated Collagen

Figure 1

Figure 2



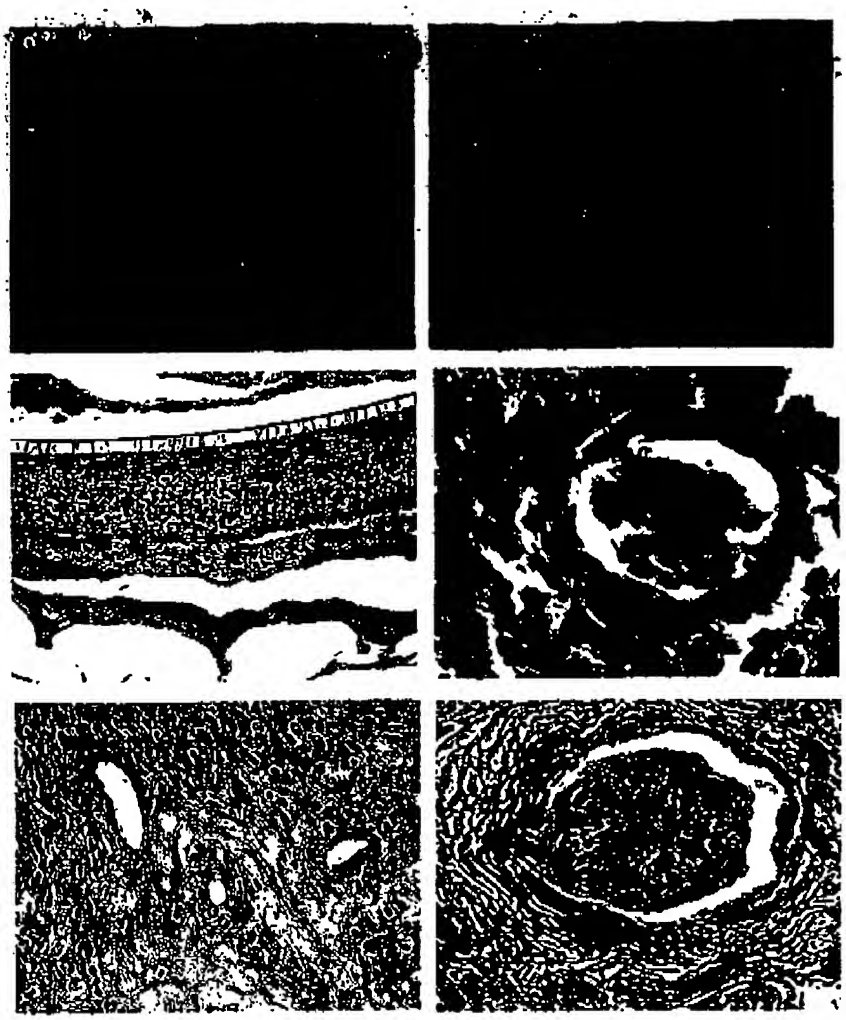
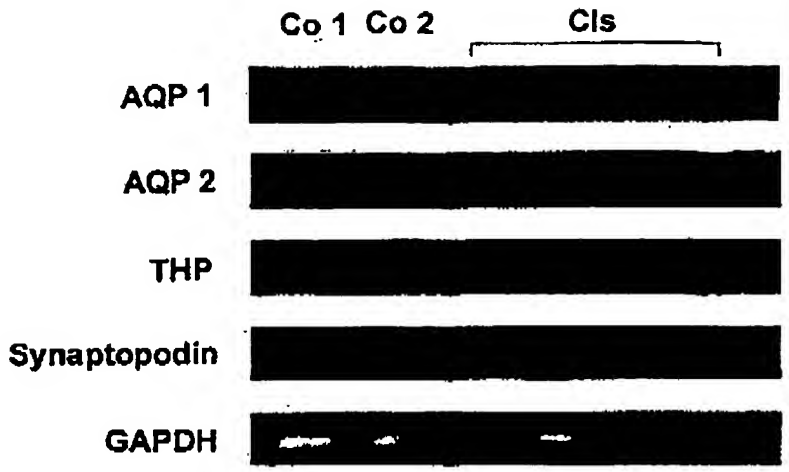


Figure 3



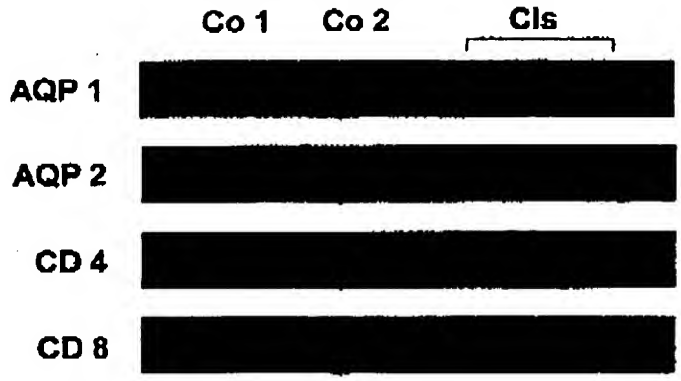


Figure 4

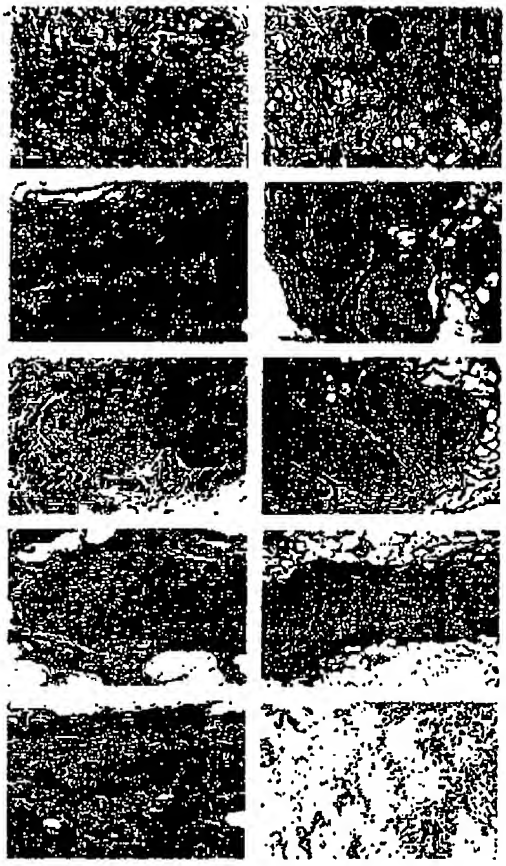


Figure 5

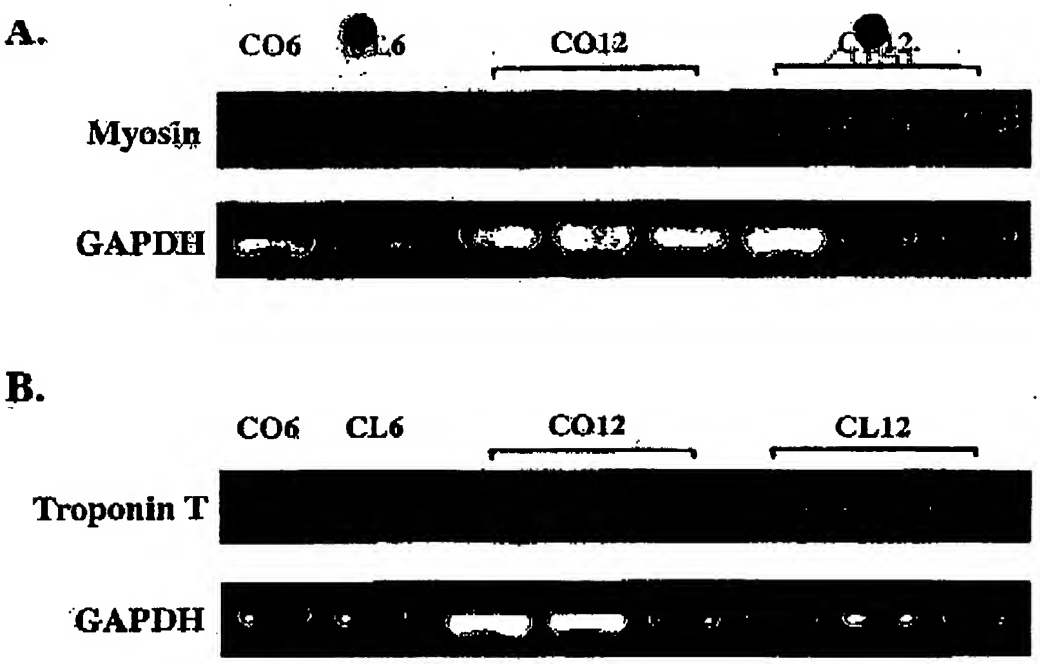


Figure 6

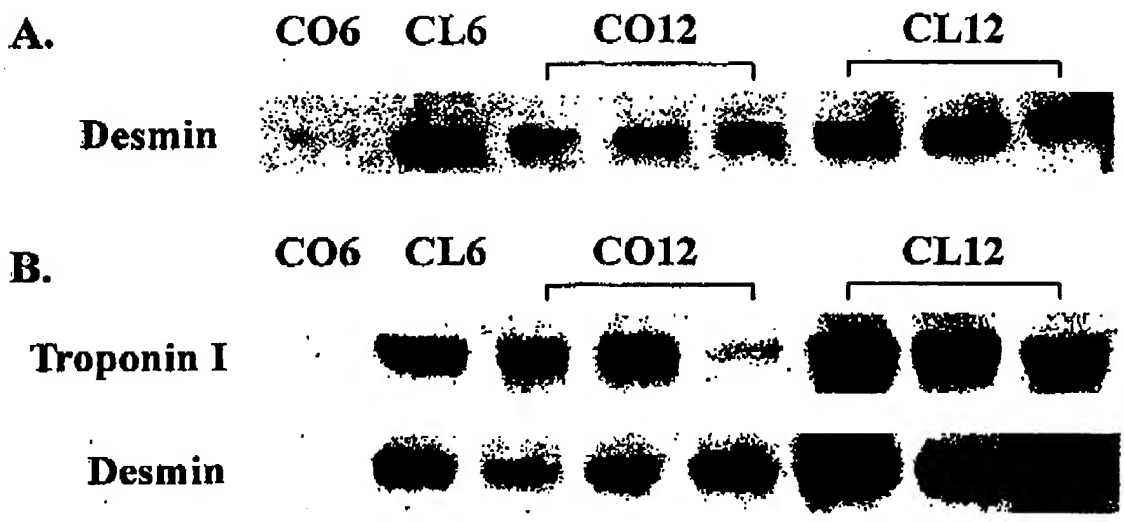


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/07109

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : G01N 33/00; A01K 67/00, 67/033, 67/027; C12N 5/00, 5/02, 15/00, 15/09, 15/63, 15/70, 15/74, 15/85, 15/87
US CL : 800/3,18,21,22,25; 435/455,463,320.1,325

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 800/3,18,21,22,25; 435/455,463,320.1,325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 2002/0046410 A1 (Lanza et al) 18 April 2002 (18.04.2002), especially entire document.	1-42
X,P	US 2002/0012655 A1 (STICE et al.) 31 January 2002 (31.01.2002), especially paragraphs [0083], [0089], [0092], [0123]-[0131], and entire document.	1-6, 15-17, 40, 41
X,P	US 6,331,406 B1 (GEARHART et al) 18 December 2001 (18.12.2001), column 3, lines 5-13, column 5, lines 1-21, column 6-7, column 10, lines 31-62.	1-6, 15-17, 40, 41
X	US 6,194,635 B1 (ANDERSON et al) 27 February 2001 (27.02.2001), column 3, lines 1-38, column 6-7.	1-6, 15-17
X	US 6,090,622 A (GEARHEART et al) 18 July 2000 (18.06.2000), column 3, lines 1-9, column 6-7, column 10, lines 22-50.	1-6, 15-17, 40, 41
X	US 5,945,577 A (STICE et al) 31 August 1999 (13.08.1999), column 4-5, column 6, lines 60-67, column 7, lines 40-55, column 8, lines 9-23, column 12, lines 43-60, column 14, column 15, lines 26-38.	1-6, 8, 15-17, 18-21, 23, 27-29, 36-38
X --- Y	US 5,942,435 A (WHEELER et al) 24 August 1999 (24.08.1999), column 3, lines 55-60, column 17, lines 9-22, column 19, lines 15-25.	1-6, 8, 9, 15-17-24, 27-29, 36-38, 40 --- 30-35

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 May 2002 (09.05.2002)	31 MAY 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Thaian N. Ton Felicia D. Roberts for Telephone No. 703-308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/07109

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	KLUG, M.G. et al. Genetically Selected Cardiomyocytes from Differentiating Embryonic Stem Cells Form Stable Intracardiac Grafts. J. Clin. Invest. 1 July 1999, Vol. 98, No. 1, pages 216-224, especially Abstract and p. 222-223.	1-8, 15-17, 22, 36, 38-41 --- 30-35

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/07109

Continuation of B. FIELDS SEARCHED Item 3:
CAPLUS, MEDLINE, EMBASE, BIOSIS, LIFESCI, WEST
search terms: stem cell, differentiate, marker, transfect, transplant, tissue, human, implant

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